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Phase Separation, Ion Permeability, and the Isolation of Membranes from Osmotically Stable Mycoplasmas

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The osmotic stability of *M. gallisepticum* was found to be a consequence of the synthesis of disaturated phosphatidylcholine incorporated into the cell membrane. The disaturated lipid induces the formation of segregated lipid domains, thus providing the sites for increased permeation of ions. Such permeation reduces the internal pressure so as to minimize cell swelling and subsequent lysis in a hypotonic medium. Purified membranes of *M. gallisepticum* can be prepared from cells suspended in an iso-osmotic NaCl solution containing either dicyclohexylcarbodiimide (DCCD), which blocks ATPase activity, or a mild alkaline buffer. Both conditions seem to interfere with cell volume regulation. These procedures can be used also to isolate membranes of other osmotically stable mycoplasmas.

INTRODUCTION

Mycoplasmas lack a rigid cell wall and are bound by a single membrane [1]. Thus, most mycoplasmas act as ideal osmometers, responding to changes in the osmotic pressure of the surrounding medium [2,3]. Yet a few species, such as *Mycoplasma gallisepticum, M. hominis,* and *M. pneumoniae* were found to be much more resistant to osmotic changes [1,4]. Due to their osmotic stability, the use of these organisms in membrane studies has been hampered by the absence of an efficient method to isolate purified membrane preparations. We present here studies on the molecular basis for the unusual osmotic stability of *M. gallisepticum* and describe gentle methods for the preparation of high yields of purified membranes from osmotically stable mycoplasma species.

MATERIALS AND METHODS

Growth of the Organism and Osmotic Fragility Measurements

M. gallisepticum (Strain A5969) was grown in a modified Edward medium [5] containing either 4 percent horse serum or 0.5 percent bovine serum albumin, 20 μg/ml cholesterol, and oleic and palmitic acids (10 μg/ml of each). For nuclear magnetic resonance (NMR) spectrometry the medium was supplemented with 10 μg/ml of phosphatidylcholine enriched in the N-methyl carbon with ^{13}C (kindly provided by B. Sears, The National Magnet Laboratory, M.I.T.). The cells were grown for 18-26 hours at 37°C, harvested, and washed as described before [5]. Osmotic behavior of the washed cells was determined by incubating the cells in 0.25 M NaCl solutions at 37°C for 15 minutes. The absorbance changes of the cell suspensions were followed at 500 nm.

Membrane Isolation

Membranes were isolated by lysing *M. gallisepticum* cells (2 mg protein/ml) in an iso-osmotic Tris-NaCl buffer (0.20 NaCl in 0.05 M Tris pH = 7.4) containing 50–100 μM dicyclohexylcarbodiimide (DCCD). The stock solution of DCCD (100 mM in dimethylsulfoxide) was freshly prepared before use. Cell lysis was usually accomplished within 15–30 minutes of incubation at 37°C. For alkaline-induced lysis, late exponential phase cells (2 mg cell protein/ml) were incubated in Tris-NaCl buffer adjusted to pH 8.5 for 60–90 minutes at 37°C. Membranes were also obtained by ultrasonic treatment [6]. All membrane preparations were collected by centrifugation at 34,000 *g* for 30 minutes, washed once, and resuspended in 0.25 M NaCl solution. The density of the membrane preparations was determined as described previously [7].

Analytical Methods

Freeze-fracturing was performed as previously described [8]. The cells, suspended in a 0.25 M NaCl solution containing 30 percent glycerol, were incubated at 4 or 37°C for one hour and then treated with 2 percent glutaraldehyde for one hour. Finally, they were frozen in liquid Freon. The samples were fractured at −110°C, replicated with platinum carbon, and examined with an electron microscope.

NMR measurements were conducted at 23°C, using a Jeol PS-100P/EC-100 Fourier transform spectrometer operating at 25.1 MHz [9].

To analyze the potassium content of intact cells, cells (containing 1 to 5 mg of cell protein) were extracted by boiling with 1 ml of 0.1 M HCl for 10 minutes. The cell residue was removed by centrifugation, and potassium content in the supernatant fluid was determined by atomic absorption spectrometry.

ATPase activity was determined by measuring the release of P_i from ATP [10] and NADH dehydrogenase activiy was assayed spectrophotometrically as previously described [11]. Protein was determined according to the method of Lowry et al. [12]. Lipids were extracted by the method of Bligh and Dyer [13]. Total phosphorus in lipids was determined according to Ames [14] after digesting the sample with ethanolic $Mg(NO_3)_2$. Cholesterol was determined by the method of Rudel and Morris [15].

RESULTS AND DISCUSSION

The Molecular Basis for the Osmotic Stability in M. gallisepticum

Many mycoplasmas incorporate large amounts of phosphatidylcholine into the cell membrane upon growth in an ordinary medium which contains horse serum [16,17]. Such incorporation by *M. gallisepticum* cells is indicated by the pronounced broadening of the nuclear magnetic resonance spectrum of ^{13}C-phosphatidylcholine added to the medium as sonicated vesicles (Fig. 1) as well as by quantitative analysis of membrane lipids [16]. In most mycoplasma species grown in medium containing horse serum, the exogenous phosphatidylcholine is incorporated unchanged into the cell membrane. Several species, however, including *M. gallisepticum* and, to a lesser extent, *M. pneumoniae* and *M. pulmonis,* modify the exogenous phosphatidylcholine by inserting a saturated fatty acid at position 2 of lysophosphatidylcholine by what appears to be a deacylation-acylation enzymatic sequence [6,17] catalyzed by a phospholipase A_2 activity present in the cell membrane. In *M. gallisepticum,* the disaturated phosphatidylcholine synthesized may reach 20–35 percent of the

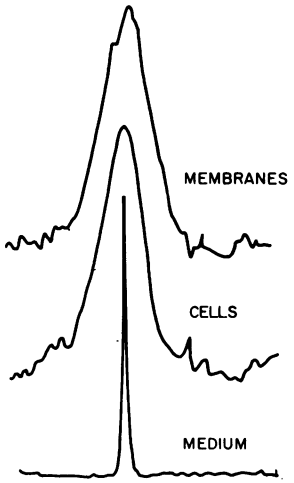

FIG. 1. NMR peak of the ^{13}C-enriched N-methyl carbon of phosphatidylcholine in the growth medium and after incorporated into *M. gallisepticum* cells and membranes.

total membrane lipids and induces the appearance of particle-free patches that are observed by freeze-fracture electron microscopy (Fig. 2). The extensive patching occurred even with membranes quenched from 37°C [8] that contained a high cholesterol-to-phospholipid molar ratio (0.9 mol cholesterol per mol of lipid phosphorus). These particle-free patches indicate the existence of segregated lipid domains in the membrane of *M. gallisepticum,* a cholesterol-poor phospholipid domain capable of undergoing order-disorder transition, and a cholesterol-rich lipid domain which remained in a fluid state and contained the intramembranous particles.

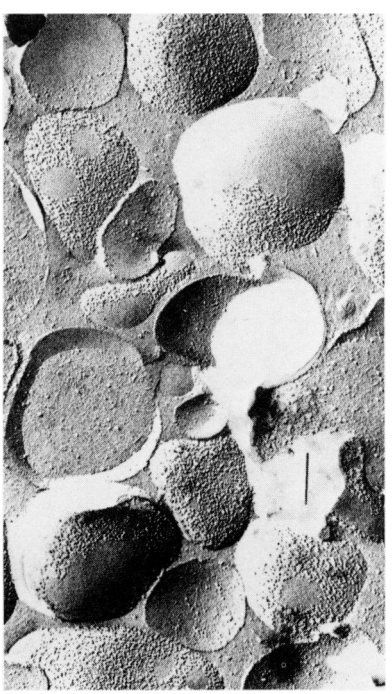

FIG. 2. Replica of freeze-fractured *M. gallisepticum* cells grown in medium containing horse serum. Bar = 0.2 μm.

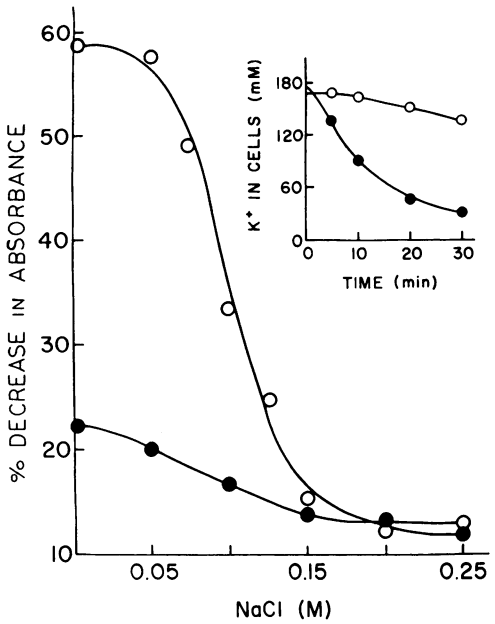

FIG. 3. Swelling of *M. gallisepticum* cells grown with (●) or without (○) horse serum, as determined by measuring the changes in absorbance of the cell suspensions at 500 nm. *Insert:* The decrease in intracellular K^+ with time in *M. gallisepticum* cells suspended in 0.05 NaCl solution.

The coexistence of fluid and solid domains within a bilayer results in disordered boundaries at the interfacial regions. These regions, in model membranes, have been associated with an increased permeability to ions and small molecules [18]. Consistent with this are our findings in *M. gallisepticum* cells correlating the existence of segregated lipid domains in the membrane with a decreased K^+ content and increased osmotic stability of the cell (Fig. 3). The segregated lipid domains that are present in *M. gallisepticum* grown on horse serum are not observed in the absence of an exogenous phospholipid source [8]. The leakage of small molecules from these cells containing segregated lipid domains reduces the internal pressure, thus minimizing osmotic swelling and subsequent cell lysis.

Cell Volume Regulation in Mycoplasma

An osmotic problem arises due to the permeability of cells to ions. Evolution has provided two solutions that give long-term stability in the face of such an osmotic crisis. One was the development of a rigid cell wall. Such a cell wall is absent in mycoplasmas. The other was specific ion pumps which utilize metabolic energy to actively extrude those salts which diffuse into the cells [19]. What is the nature of such ion pumps in mycoplasmas? In animal cells, the action of the sodium potassium ATPase extrudes Na^+ electrogenically and cells shrink due to the associated osmotic movement of water. In mycoplasma species the ionic pumping events are apparently different, since the unabain-sensitive Na^+-K^+-ATPase is not present. An alternative mechanism for Na^+ extrusion found in many microorganisms is the pumping of protons via the H^+-ATPase and extruding Na^+ by means of the H^+-Na^+ exchange mechanism. The diagram given in Fig. 4 illustrates this mechanism. Our recent findings that energy-depleted *M. gallisepticum* cells swell when suspended in iso-osmotic NaCl solution and that the swelling can be prevented by the addition of glucose [20] support such a hypothesis.

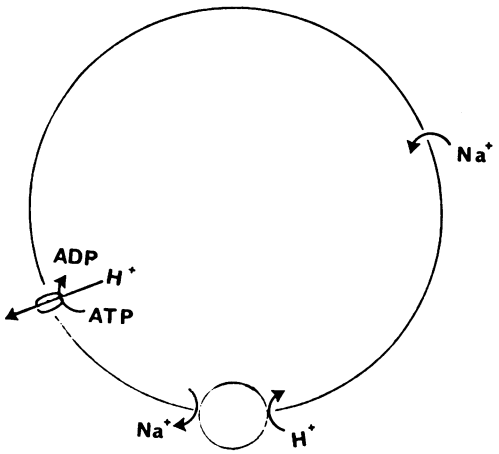

FIG. 4. Schematic representation of a sodium pump driven by a proton gradient which in turn results from a ATP-driven proton pump. (Redrawn from Wilson and Lin [20]).

Isolation of Mycoplasma Membranes by Interfering with Cell Volume Regulation Mechanism

It seems that an energy-dependent process operated via a membrane ATPase and a Na^+-H^+ exchange carrier are involved in cell volume regulation of *M. gallisepticum,* inhibiting the ATPase activity or interfering with the Na^+-H^+ exchange carrier of cells suspended in an iso-osmotic NaCl solution. It will result in a pronounced increase in the NaCl and water entering the cell by diffusion. This may cause swelling of the cell and its subsequent lysis. We therefore attempted to obtained purified membrane preparations of *M. gallisepticum* cells either by the use of DCCD [22], a specific inhibitor of proton-translocating ATPases, or by treating non-energized cells at the alkaline pH range, at which the external proton concentration is very low. In both procedures cell lysis was most pronounced with cells grown in serum containing media. These cells synthesize the disaturated phosphatidylcholine [6] and are more permeable to ions and small solutes. The age of the culture did not decrease the ability of DCCD to induce lysis, whereas the mild alkaline treatment was more efficient with late exponential or stationary phase cells. Thin section electron microscopy of the isolated *M. gallisepticum* obtained by both methods revealed homogenous arrays of vesicles relatively free of cytoplasmic contaminants [20,21]. Table 1 presents various properties of membranes obtained by DCCD lysis, mild alkaline-induced lysis, and ultrasonication. The lipid-to-protein ratio was considerably higher in DCCD membranes and membranes obtained by alkaline-induced lysis than in membranes obtained by ultrasonication. This ratio was about 3.5 times higher than in intact cells, with >95 percent of cell lipids retained in the membranes. The lipid-to-protein ratio was consistent with the sucrose density gradient analysis showing densities of 1.17 and 1.18 g/ml for DCCD membranes and membranes obtained by mild alkaline treatment, respectively. Such densities fell within the density range reported for mycoplasma membranes obtained by osmotic lysis [1,15] and were considerably higher than the density of *M. gallisepticum* membranes obtained by ultrasonic treatment. The membranes isolated either by the DCCD procedure or by mild alkaline lysis were without appreciable contamination of cytoplasmic components as judged by the lack of NADH dehydrogenase activity, which in mycoplasmas is a soluble enzyme [1,4]. Membranes obtained by the DCCD pro-

TABLE 1
Properties of *M. gallisepticum* Cell and Membrane Preparations

Preparation	Lipid-to-Protein Ratio (nmoles/mg protein)		Density (g/ml)	Enzymatic Activity[a]	
	Phospholipids	Cholesterol		NADH Dehydrogenases	ATPase
Intact Cells	68	60	1.28	13.6	82
Isolated Membranes Obtained by:					
Ultrasonic Treatment	150	138	1.21	1.8	203
DCCD Lysis	256	232	1.17	0.1	87
Alkaline-Induced Lysis	240	230	1.18	0.1	104

[a]NADH dehydrogenase activity was expressed as decrease in absorbance at 340 nm per milligram protein per minute. ATPase activity was expressed as nanomoles inorganic phosphate released per milligram of protein per minute.

cedure have, however, the disadvantage of having a lower ATPase activity and retaining the DCCD, which is highly hydrophobic.

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